Sclerostin failed to restore the expression of the anabolic genes, we investigated the effect of Sclerostin on the activation of Wnt non canonical pathways mediated by Ca2+/CaMKII, JNK and PKC proteins.
Methods: Primary murine chondrocytes were cultured with or without Wnt3a conditioned media and in the presence or absence of recombinant Sclerostin. Activation of Ca2+/CaMKII, JNK and PKC pathways was analyzed by Western blotting. The role of the activation of JNK and PKC pathways in the chondrocyte phenotype was investigated using SP600125 and Staurosporin inhibitors respectively. Chondrocyte differentiation was investigated by RT-qPCR through the relative expression of type II and X collagen, Sox9, Aggrecan, MMP-3 & 13 and ADAMTS-4&5 genes and by Western blotting through the protein expression of ADAMTS-4 & 5 and type X Collagen Alcian blue staining and spectrophotometric quantification was also used for analyzing the accumulation of highly sulphated GAG. .
Results: Wnt3a increased the gene expression of metalloproteinases such as Adamts-4 & 5 MMP3, 13 and type X collagen. This effect was totally abolished in the presence of Sclerostin. In the other hand, Sclerostin restored partially the expression of type II Collagen, Sox9 and Aggrecan inhibited by Wnt3a. Because of the partial effect of Sclerostin on the anabolic genes, we assessed whether Wnt3a activates β-catenin independent pathways such as JNK, PKC and CaMKβII. Wnt3A promoted the phosphorylation of JNK and PKC without affecting CaMKβII phsphorylation. Moreover, Sclerostin inhibited the phosphorylation of JNK but not that of PKC. We further investigated whether Sclerostin-induced inhibition of JNK affects chondrocyte function. We found that Wnt3a decreased the accumulation of highly sulphated GAG while Sclerostin failed in rescuing their amount. Interestingly, Sclerostin was able to rescue the accumulation of highly sulphated GAG and the expression of the anabolic genes when JNK pathway is inhibited by SP600125.
Conclusions: We here showed that Wnt3a inhibits the expression of the anabolic genes by activating the non-canonical JNK pathways in chondrocytes. Sclerostin reduced the Wnt-induced activation of JNK pathway. These results show that Sclerostin may play a role in the anabolic activity of chondrocytes.

221
DIFFERENTIAL EFFECTS OF BONE MORPHOGENETIC PROTEIN 2 AND 9 ON CHONDROPROTECTIVE TRANSFORMING GROWTH FACTOR B SIGNALING

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Purpose: Osteoarthritis (OA) is a multifactorial disease characterized by loss of articular cartilage. TGFβ is considered as a protective factor against cartilage loss in young cartilage by inducing Smad2/3 phosphorylation via the TGFβ receptor ALK5. Smad2/3 phosphorylation (Smad2/3P) lowers MMP13 expression, and prevents deleterious terminal differentiation of chondrocytes. In contrast to Smad2/3P, Smad1/5 phosphorylation, induced by BMPs but also by TGFβ, is linked to terminal differentiation and MMP13 production. Type I receptors that phosphorylate Smad1/5/8 include ALK1 and ALK3. BMP-9 signals via ALK1 and BMP-2 via ALK3, and both ligands induce enhanced glycosaminoglycan synthesis in young cartilage. BMP2 is induced in damaged cartilage while BMP9 is constitutively present in high concentrations in body fluids. If and how these factors modulate chondroprotective TGFβ signaling is still unclear. Therefore, we analyzed BMP-2 and BMP-9 induced Smad signaling and gene expression in chondrocytes, and studied their interaction with TGFβ.
Methods: Primary bovine chondrocytes, isolated from the metacarpophalangeal joint of 2 year old animals, or the human G6 chondrocyte cell line were cultured to near confluency and incubated with TGFβ1, BMP-2, BMP-9 or a combination thereof. Smad phosphorylation kinetics were analyzed by specific Smad2p and Smad1/5p staining of Western blots. Expression patterns of Smad specific response genes: PAI-1 (Smad3 signaling), and ID-1, (Smad1/5 signaling) were analyzed from 0-48h by quantitative real time PCR (qPCR). Biological activity was tested with a $CAGA_{12}$-luc transcriptional reporter construct that produces luciferase in response to Smad3 signaling. Adenovirally transduced cells (MOI = 5) were serum starved for 8h, and stimulated with growth factors for 20h, and luciferase activity was measured.
Results: In primary chondrocytes, both BMP-2 and BMP-9 potently induced Smad1/5 phosphorylation, which peeked after 1 h and lasted up to 3 h. BMP-9 was more potent than BMP-2 in inducing Smad1/5p, as a lower dose of BMP-9 (5 ng/ml BMP-9 versus 15 ng/ml BMP-2) was required to induce maximal phosphorylation after 60 min. Remarkably BMP-9 (5 ng/ml or more) was also capable of inducing Smad2 phosphorylation, whereas BMP-2 (up to 50 ng/ml) was not. BMP-9-induced Smad2 phosphorylation lasted from 1 up to 3 hours. Moreover, in contrast to BMP-2, BMP-9 was also able to rapidly (from 2 up to 24h) induce PAI-1 transcription. Both growth factors potently induced ID-1 expression. Strikingly, co-stimulation of primary chondrocytes with TGFβ (1 ng/ml) and these BMPs revealed an even more remarkable difference between both factors on Western blot. BMP-2 was able to dose-dependently (15-50 ng/ml) inhibit TGFβ-induced Smad2 phosphorylation. On the contrary, BMP-9 dose-dependently (5-25 ng/ml) synergized with TGFβ on Smad2/3 phosphorylation. These results were reflected in biological activity, as luciferase production was increased by 50% after co-stimulation of chondrocytes with BMP-9 and TGFβ compared to TGFβ alone ($p<0.01$).
Conclusions: In young cartilage, both BMPs show a distinct difference in Smad phosphorylation and interaction with TGFβ. Although both BMPs promote matrix synthesis (Glycosaminoglycans production), long term effects of both factors on cartilage will most likely differ due to their different effects on chondroprotective Smad2/3 signaling.

222
IN SITU CALCIUM SIGNALING OF CHONDROCYTES UNDER SERUM-FREE AND SERUM CULTURE

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Purpose: Previous research has shown that serum-free medium can maintain the mechanical properties of cartilage allografts better than serum supplemented medium. However, little is known about this beneficial mechanism at a cellular level. Intracellular calcium ($[Ca^{2+}]_i$) signaling, one of the earliest responses in chondrocytes under mechanical stimulation, can regulate the differentiation and metabolism of cells. Therefore, we hypothesized that the beneficial mechanisms of serum-free culture could be reflected by the spatiotemporal properties of $[Ca^{2+}]_i$ signaling of chondrocytes. We aimed to: (i) record, analyze and compare the *in situ* spontaneous $[Ca^{2+}]_i$ responses of chondrocytes cultured in serum-free and serum medium, and (ii) compare the biomechanical properties of cartilage explants under two culture conditions.
Methods: Cylindrical cartilage allografts were harvested from femoral condyle head of calf knee joints. Superficial zone of the cartilage (2mm in thickness) were cultured in either serum medium (DMEM+10% FBS) or serum-free medium (Bian L., et al., 2008) for 1 month. Spontaneous $[Ca^{2+}]_i$ responses and mechanical properties were recorded at day 2, 8, 15, and 29. After cartilage allograft was cut into identical halves and dyed with Fluo-8 AM, calcium images were recorded with a confocal microscope (Fig. 1A). Spatiotemporal parameters of the $[Ca^{2+}]_i$ spikes were extracted by imaging processing. The dynamic modulus of allografts was tested at 0.5 Hz and 1% strain with unconfined compression.
Results: A typical fluorescent image of *in situ* chondrocytes is shown in Fig. 1B. Responsive percentage of cells in serum culture decreased with culture time, while the serum-free group showed no statistical difference within 1 month (Fig. 2A). Average magnitude of $[Ca^{2+}]_i$ peaks (Fig. 3A) in serum-free group was lower than that in serum group at day 2 but significantly higher afterwards. Average number of $[Ca^{2+}]_i$ peaks for responsive cells in serum-free group was significantly lower than that in serum group at day 2, but higher after day 15 (Fig. 2B). Time interval between two neighboring peaks is significantly longer in serum group at day 29 (Fig. 3D). Peak relaxation time is slightly longer in serum-free group at day 1, but shorter after day 8 (Fig. 3C). Dynamic modulus of explants in serum-free group was significantly higher than those in serum group after day 8.
Conclusions: 1) At day 2, more chondrocytes in serum culture medium showed spontaneous $[Ca^{2+}]_i$ peaks, with higher magnitudes and faster responsive speed than those in serum-free medium. However, this trend turned opposite after 8-day culture. 2) Serum culture benefits the

spontaneous calcium signaling of chondrocytes in short-term, but serum-free culture can better maintain calcium responses of chondroctyes in long-term culture. 3) Serum-free culture can significantly benefit the dynamic properties of cartilage compared with serum culture. These results imply that serum-free culture can maintain regular biochemical activities of chondrocytes better than serum medium, which may further benefit the biomechanical properties of cartilage explants.

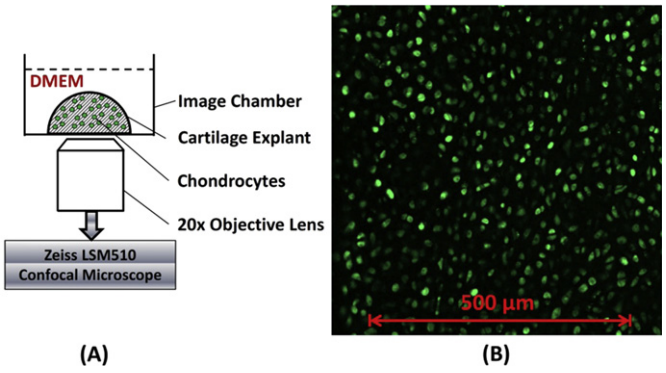

Fig. 1. (A) Cartilage explant on the confocal microscope; (B) A fluorescent image of chondrocytes in cartilage explant.

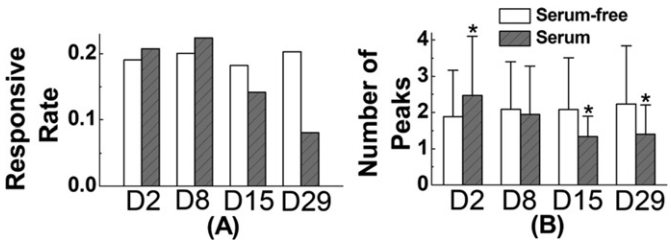

Fig. 2. Comparison of $[Ca^{2+}]_i$ responses between serum and serum-free group (serum-free group: 4068 cells, serum group: 3444 cells). (A) Responsive percentage of cells; (B) Number of peaks of responsive cells. (*: $p<0.05$, between the two groups).

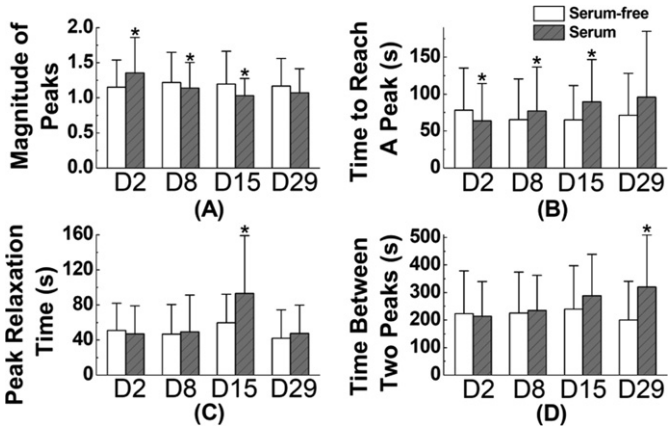

Fig. 3. Comparison of spatiotemporal properties of $[Ca^{2+}]_i$ responses. (A) Magnitude of all peaks for each cell (arbitrary unit); (B) Time to reach a calcium peak; (C) Peak relaxation time; (D) Time interval between two peaks. (*: $p<0.05$, between the two groups).

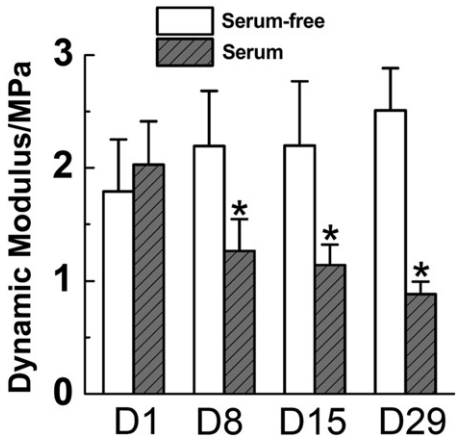

Fig. 4. Dynamic modulus of cartilage in serum and serum-free group (n=12). (*: $p<0.05$, between the two groups).

223
ELEVATED HEPATOCYTE GROWTH FACTOR LEVELS IN OSTEOARTHRITIS OSTEOBLASTS CONTRIBUTE TO THEIR ALTERED RESPONSE TO BONE MORPHOGENETIC PROTEIN-2 AND REDUCED MINERALIZATION

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Purpose: Clinical and *in vitro* studies suggest that subchondral bone sclerosis due to abnormal osteoblasts (Ob) is involved in the progression and/or onset of osteoarthritis (OA). Human Ob isolated from sclerotic subchondral OA bone tissue show an altered phenotype, a decreased canonical Wnt/ß-catenin pathway (cWnt), and a reduced mineralization *in vitro* as *in vivo*. These alterations were linked with an abnormal response to BMP-2. OA Ob release factors such as the Hepatocyte Growth Factor (HGF) that contribute to cartilage loss whereas chondrocytes do not express HGF. A paracrine cross-talk between the subchondral bone compartment and articular cartilage may occur during OA via HGF. HGF can stimulate BMP-2 expression in human Ob, however, the role of HGF and its effect in OA Ob remains unknown. Here we investigated whether HGF in OA Ob is responsible for the altered response to BMP-2.

Methods: We prepared primary human subchondral osteoblasts using the sclerotic medial portion of the tibial plateaus of OA patients undergoing total knee arthroplasty, or from tibial plateaus of normal individuals at autopsy. The expression of HGF was evaluated by qRT-PCR and the protein production by Western blot analysis. HGF expression was reduced with siRNA technique whereas its activity was inhibited using the selective inhibitor PHA665752. Alkaline phosphatase activity (ALPase) and osteocalcin release (OC) were measured by substrate hydrolysis and EIA respectively. Canonical Wnt/β-catenin signaling (cWnt) was evaluated using two approaches: 1) target gene expression was measured using the TOPflash TCF/lef luciferase reporter assay, and 2) intracellular signaling partners β-catenin and phospho β-catenin levels were evaluated by Western blot analysis. Mineralization in response to Wnt3a was evaluated by Alizarin red staining.

Results: The expression of HGF was increased in OA Ob compared to normal Ob and OA Ob released more HGF as assessed by Western blot analysis. Transforming growth factor β-1 (TGF-β1) inhibited this expression in all cells. Conversely, HGF stimulated the expression of TGF-β1 when terminal differenciation of OA Ob was triggered with vitamin D_3. BMP-2 dose-dependently (1 to 100 ng/ml) stimulated both ALPase and OC in normal Ob whereas it inhibited them in OA Ob. Using HGF-siRNA treatments reversed this response in OA Ob and restored the BMP-2 response. cWnt was reduced in OA Ob compared to normal and HGF-siRNA treatments increased cWnt in OA Ob almost to normal. Smad 1/5/8 phosphorylation, which is reduced in OA Ob, was corrected with prior addition of PHA665752 to these cells. The BMP-2-dependent